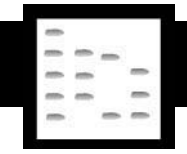
ALKALOIDS



Gas Chromatography

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Introduction

Alkaloids are an important class of compounds that have pharmacological effects on the human body. These compounds can be found in natural products such as plants, and the type and amount of these alkaloids varies greatly, depending on the portion of plant analysed and the stage of maturation. Although alkaloids have traditionally been isolated from plants, an increasing number are to be found in animals, insects, marine invertebrates and microorganisms.

There is no clear definition of what constitutes an alkaloid, but these compounds do share the following characteristics: they are basic components that contain nitrogen; they are mostly complex components, derived biosynthetically from various amino acids; and they show pronounced pharmacological effects on various tissues and organs of humans and other animal species.

Pelletier defines an alkaloid as 'a cyclic compound containing nitrogen in a negative oxidation state which is of limited distribution in living organisms'. This definition includes both alkaloids with nitrogen as part of a heterocyclic system as well as the many exceptions with extracyclic bound nitrogen (**Figure 1**).

Although a wealth of information is available on the pharmacological effects of these compounds, little is known about how plants synthesize these substances or about how this synthesis is regulated. Alkaloids belong to the broad category of secondary metabolites. This class of molecule has historically been defined as a naturally occurring substance that is not vital to the organism that produces them. Alkaloids have traditionally been of interest only due to their pronounced and various physiological activities in animals and humans. A picture has now begun to emerge that alkaloids do have important ecochemical functions in the defence of the plant against pathogenic organisms and herbivores and are found to play an important role in plant interactions with animals and higher and lower plants. Alkaloids are now generally considered to be part of an elaborate system of chemical defence in plants; indeed, the same seems to be true in vertebrates, invertebrates and microorganisms. Alkaloids have now been isolated from such diverse organisms as animals, insects, marine organisms, microorganisms and lower plants, although it is not yet clear whether *de novo* alkaloid biosynthesis occurs in each organism.

In the past ten years there has been an increasing interest in the isolation and determination of alkaloids in plant materials, in pharmaceutical products, and in other samples of biological interest. In addition, numerous alkaloids have been synthesized and chemically characterized. The active agents of around 13 000 plant species are known to have been used as drugs throughout the world. Some are used as pure compounds for therapeutic purposes (such as the narcotic and analgesic, morphine; the analgesic and antitussive, codeine; and the chemotherapeutic agents, vincristine and vinblastine) or as teas and extracts. Plant constituents have also served as models for modern synthetic drugs, such as atropine for tropicamide, quinine for chloroquine, and cocaine for procaine and tetracaine. Alkaloids can also be found in the stimulants caffeine in coffee and tea and nicotine in cigarettes. Currently, much work is being done to discover new alkaloid molecules for different applications such as new antiviral and tumour treatments.

However, many alkaloids are toxic substances and it is important to evaluate these. The vegetables Solanaceae, which contain steroidal glycoalkaloids, and Leguminosae, which contain quinolizidine alkaloids, are the principal food crops that contain alkaloids. Grain legumes are extremely important owing to their significance in human and animal nutrition. They also conserve the soil and fix nitrogen, and are used as sources of timber, fuel oils, etc. Plants of the Leguminosae rank second in economic importance only to those of the Gramineae, and the demand for legumes is likely to escalate as humans begin to utilize more marginal agricultural lands to provide food for the increased population. The largest legume subfamily is the Papilionaceae, which embraces approximately 440 genera and 12 000 species in 32 tribes, as recently reclassified by Polhill. Over 450 alkaloids have been reported to occur in plants of the Leguminosae, with the majority of such compounds occurring in papilionaceous species. Quinolizidine alkaloids (QA), contained in lupins, are the largest single group of legume alkaloids. Since lupin seeds contain

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up to 50% protein and up to 20% lipids, they are of interest in terms of animal and human nutrition. *Lupinus luteus*, *L. albus* and *L. angustifolius* have been consumed for centuries in European countries, while *L. mutabilis* (tarwi) is an important component of the South American diet.

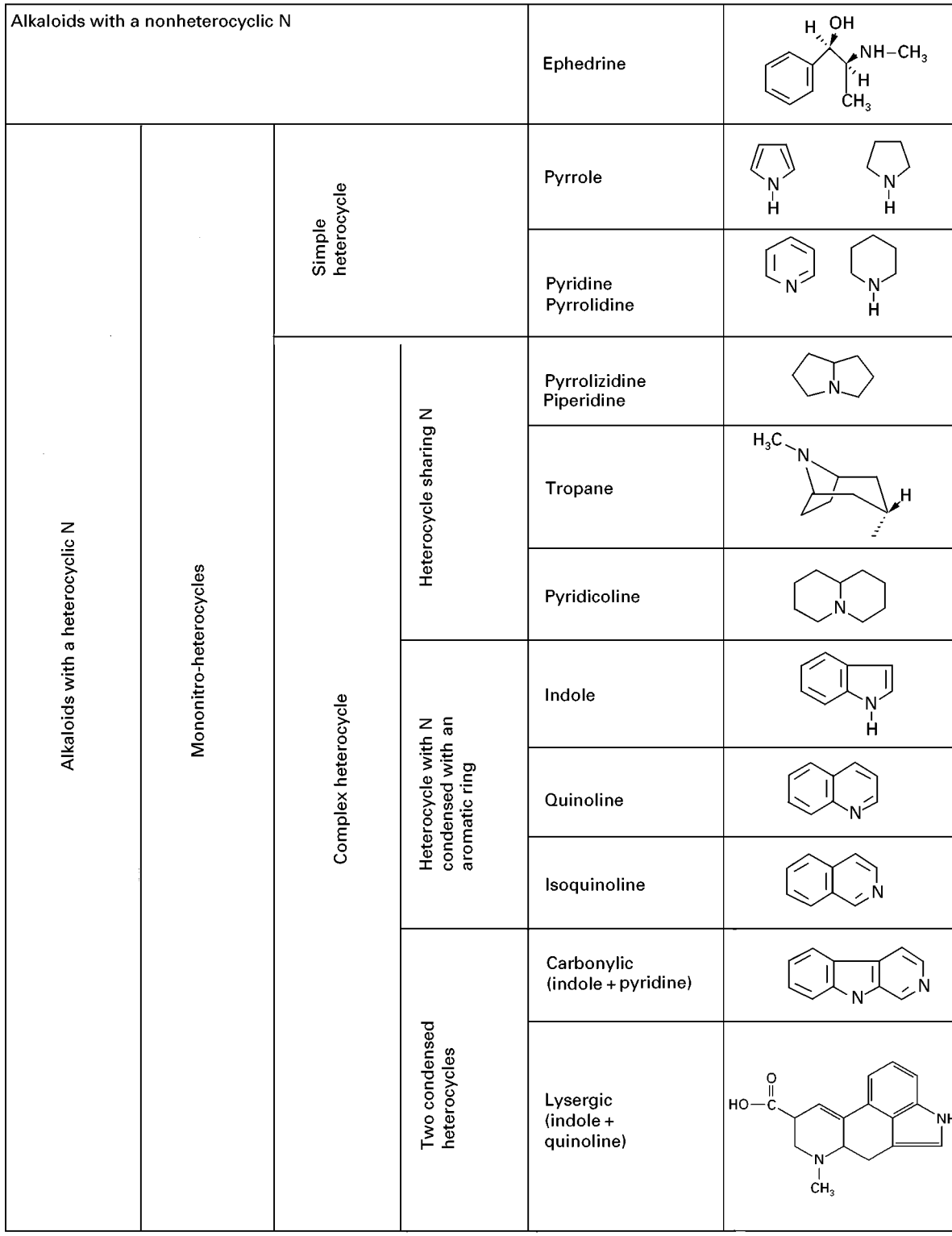

Figure 1 Chemistry classification of alkaloids.

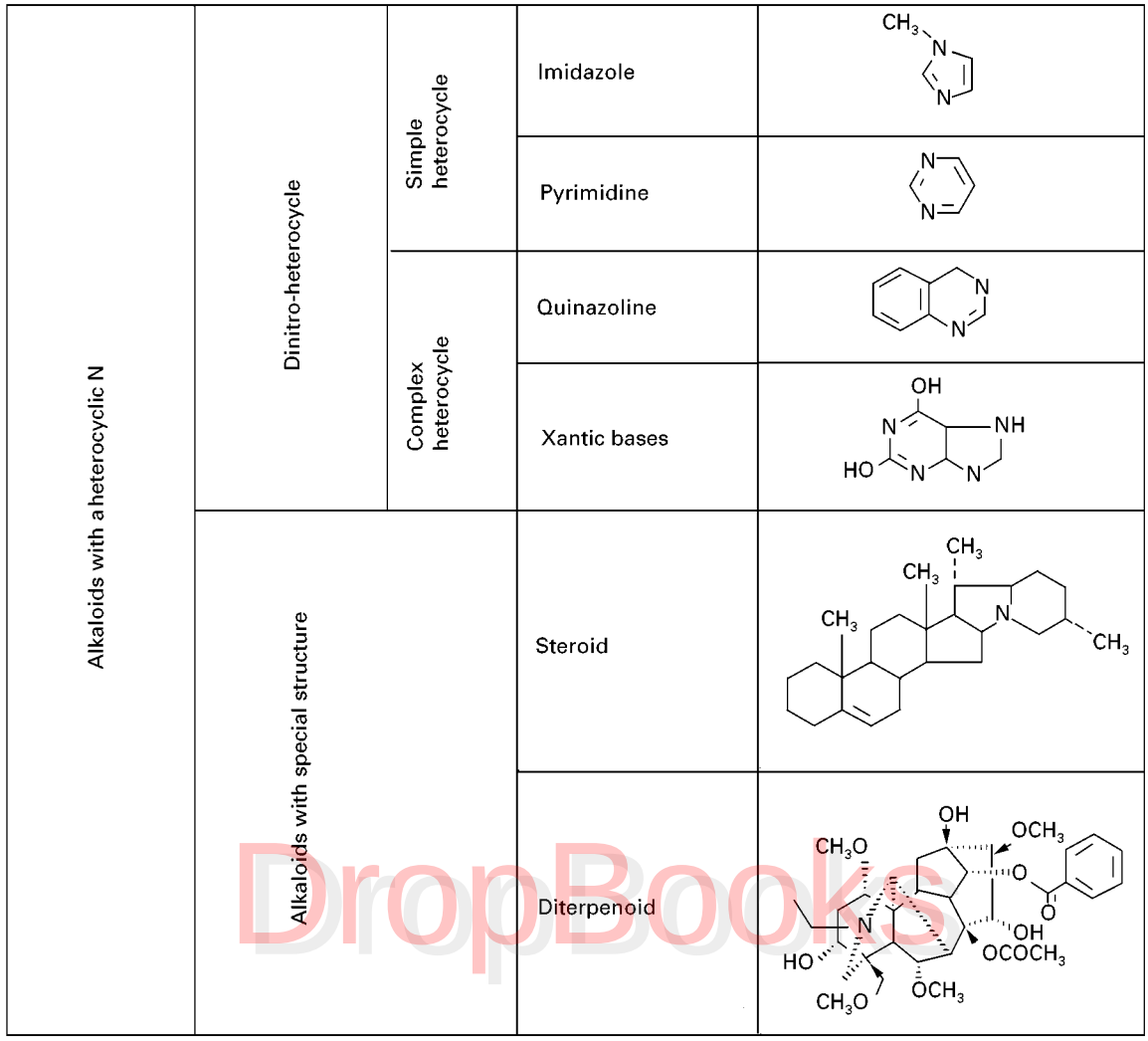

Figure 1 *Continued*

This article aims to provide an overview of various aspects of separation of alkaloids by gas chromatography (GC). Although a number of phytochemical methods have been developed for the qualitative and quantitative determination of alkaloids, one of the most popular methods for the evaluation of complex alkaloid mixtures is capillary gas–liquid chromatography combined with mass spectrometry (MS). Depending on the task high performance liquid chromatography (HPLC), thin-layer chromatography (TLC), colorimetry, NMR, radioimmunoassay, capillary electrophoresis and enzyme-linked immunosorbent assay (ELISA) are additional helpful analytical techniques.

GC-MS Method for Analysis of Alkaloids

Capillary gas chromatography (CGC) analysis has been described for several classes of alkaloids. A major advantage of GC over other methods is its enhanced sensitivity and high resolution. Another advantage is its easy coupling to a mass spectrometer, which allows the identification of new and minor compounds of a mixture without laborious isolation procedures. This makes it a particularly attractive method for thermally stable mixtures. The analysis of pyrrolizidine alkaloids, tropane alkaloids, steroidal alkaloids, quinazoline alkaloids, quinolizidine alkaloids, diterpenoid alkaloids and lycopodium alkaloids has been described by a number of authors.

Capillary gas chromatography was the method of choice and was officially accepted at the 6th International Lupin Conference in Chile (1990) as a method of determination of quinolizidine alkaloids in lupins. As an example, we will describe the methodology for the analysis of these compounds.

Sample Preparation for Chemical Analysis of Alkaloids

Successful chemical analysis of alkaloids depends on the sampling method and pretreatment of the sample. It is therefore important to know the chemistry of the compounds to be analysed. As described by Roberts and Wink, a basic character is no longer a prerequisite for an alkaloid and the chemistry of the nitrogen atom allows for at least four groups of nitrogenous compounds:

1. Secondary and tertiary amines, which are more or less protonated and therefore hydrophilic at pH < 7.0, or the more general case where they are lipophilic and unprotonated at pH > 8.0. This is the classical alkaloid type.
2. Quaternary amino compounds, which are very polar, charged at all pH values, and have to be isolated as salts, e.g. berberine and sanguinarine.
3. Neutral amino compounds, which include the amide-type alkaloids such as colchicine, capsaicin, and most lactams, e.g. ricinine.
4. *N*-oxides, which are generally highly water soluble, are frequently found in many alkaloid classes. The pyrrolizidine group of alkaloids is rich in this particular alkaloid type.

A conventional alkaloid extraction process involves successive removal or nonalkaloids and alkaloids by organic solvents from acidified and basified aqueous solutions of an ethanol extract. The extraction of alkaloids is generally based on the fact that they normally occur in the plant as salts and on their basicity, in other words on the differential solubility of the bases and salts in water and organic solvents (**Figure 2**).

The techniques used for sample preparation are liquid–liquid extraction, solid-phase extraction and, more recently, supercritical fluid extraction.

Liquid–liquid solvent extraction This technique is the most commonly used method for sample treatment and is based on the observation that alkaloids can usually be removed from the sample by extracting them into a water-immiscible solvent. The method relies on the relative solubility of alkaloids in the extracting solvent and the sample matrix.

Although such techniques are usually satisfactory, difficulties can be found when they are applied to chromatography where the limits of quantification are often in the ppb range. This is principally caused by the solvents being nonselective and therefore tending to extract endogenous material from the matrix, which results in spurious peaks in the chromatogram.

An example of quinolizidine alkaloid liquid–liquid extraction is provided in **Figure 3**.

Solid-phase extraction for sample preparation Sample clean-up is required when impurities in the sample matrix interfere with analyte measurement. The interest in this technique led to the commercial introduction of small disposable cartridges packed with relatively large particles of various bonded silicas. The particle size allows the use of minimum pressure to force the sample and wash solutions through the column. Indeed, it is common practice to suck the solution through the packings rather than to use pressure.

There are many advantages of solid-phase extraction including: (1) the possible use of large sample sizes in pretreatment; (2) the technique is quick and automated; (3) the low consumption of solvents used; (4) the use of selective sorbents and solvents; (5) the possible achievement of a high pre-concentration of the component of interest, enabling high sensitivities to be obtained; (6) there is good reproducibility in GC; and (7) the technique is inexpensive.

In developing assays using solid-phase extraction, it is necessary to take into account several factors when deciding on the choice of sorbent to be used in a particular assay for alkaloid analysis.

The most important consideration of the technique is that the compounds of interest must be capable of being readily absorbed from the matrix. In some cases, pretreatment of the sample is necessary, especially in cases of protein binding. This can usually be solved by the addition of perchloric or trichloroacetic acid to denature the proteins. In addition, it may be necessary to adjust the pH of the sample to ensure that the compound is in the correct ionic form to achieve efficient retention by the packing. Proteins can also be removed by the addition of organic solvents such as acetonitrile or methanol.

After removing the majority of the interfering substances, the final step of the technique is efficient elution from the bonded silica. This step must ensure that the compounds of interest are desorbed in the least volume of eluent, since it is usual to evaporate the solution to dryness and reconstitute the residue in a small volume prior to chromatographic analysis. The evaporation step generally precludes the use of inorganic salts in the final wash solution, with the exception of those compounds that are readily volatile.

Quinolizidine alkaloid solid-phase extraction is illustrated in **Figure 4**.

Analytical supercritical fluid extraction At present, and in view of increasing environmental concerns of

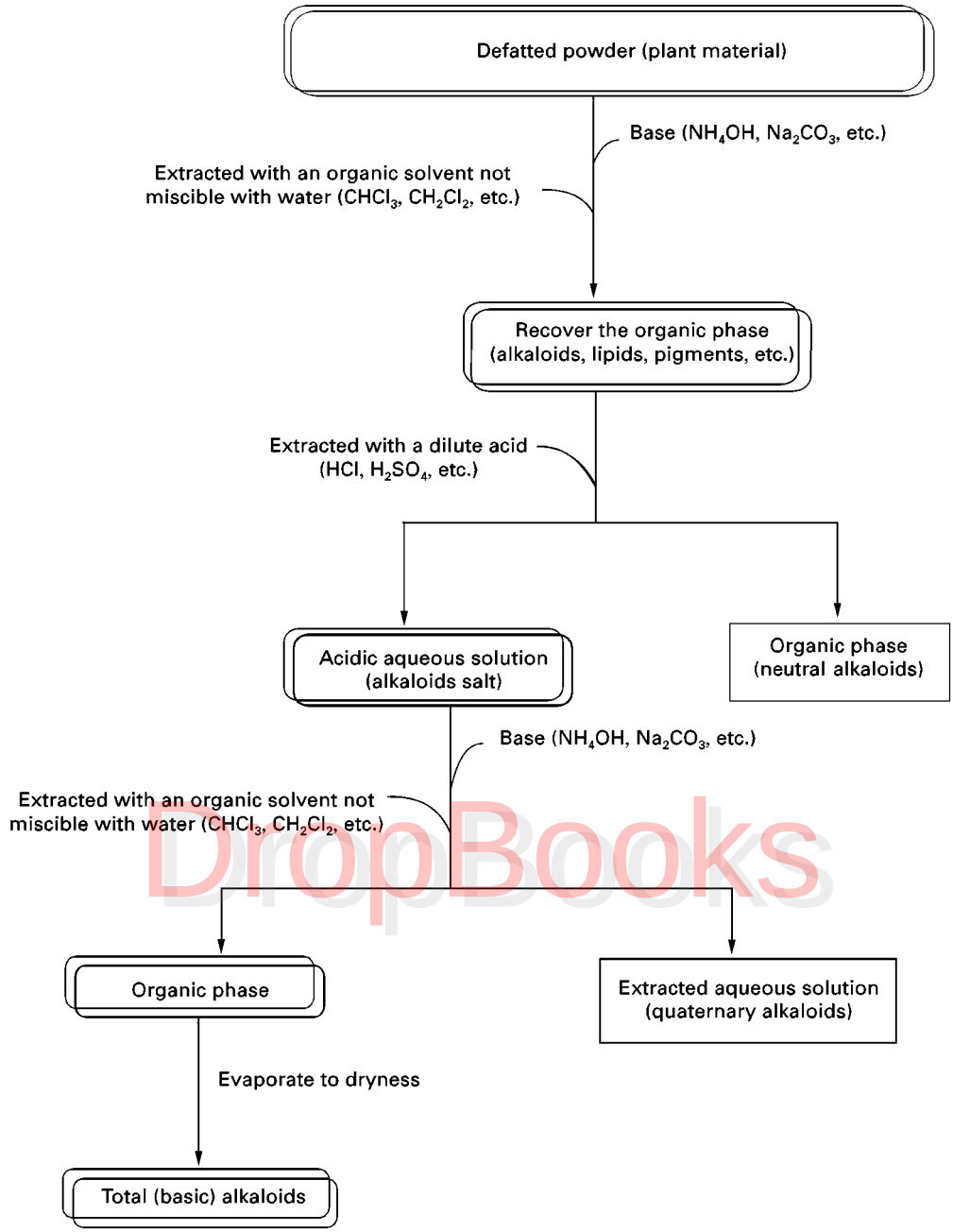
Defatted powder (plant material)
Base (NH_4OH, Na_2CO_3, etc.)
Extracted with an organic solvent not miscible with water ($CHCl_3$, CH_2Cl_2, etc.)
Recover the organic phase (alkaloids, lipids, pigments, etc.)
Extracted with a dilute acid (HCl, H_2SO_4, etc.)
Acidic aqueous solution (alkaloids salt)
Organic phase (neutral alkaloids)
Base (NH_4OH, Na_2CO_3, etc.)
Extracted with an organic solvent not miscible with water ($CHCl_3$, CH_2Cl_2, etc.)
Organic phase
Extracted aqueous solution (quaternary alkaloids)
Evaporate to dryness
Total (basic) alkaloids

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Figure 2 Extraction of alkaloids.

the use of liquid solvents in the extraction of natural products, there has been growing interest in alternative and reliable sample extraction techniques using supercritical fluids. Supercritical fluids have been widely used for the extraction of alkaloids on both analytical and industrial scales and for many years for the selective extraction of selected compounds from bulk samples. The extraction of caffeine from coffee is a well-known process performed on an industrial scale. The aim here is to remove a specific component (i.e. caffeine) from large quantities of the bulk matrix in order to increase its commercial value.

Analytical-scale supercritical fluid extraction (SFE) is concerned more with extraction of analytes of interest from a bulk matrix as a sample preparation step prior to characterization by other analytical methods such as GC. It is therefore potentially very useful for the extraction of natural products prior to structural characterization. SFE is gaining acceptance as an alternative to Soxhlet extraction. Much of the

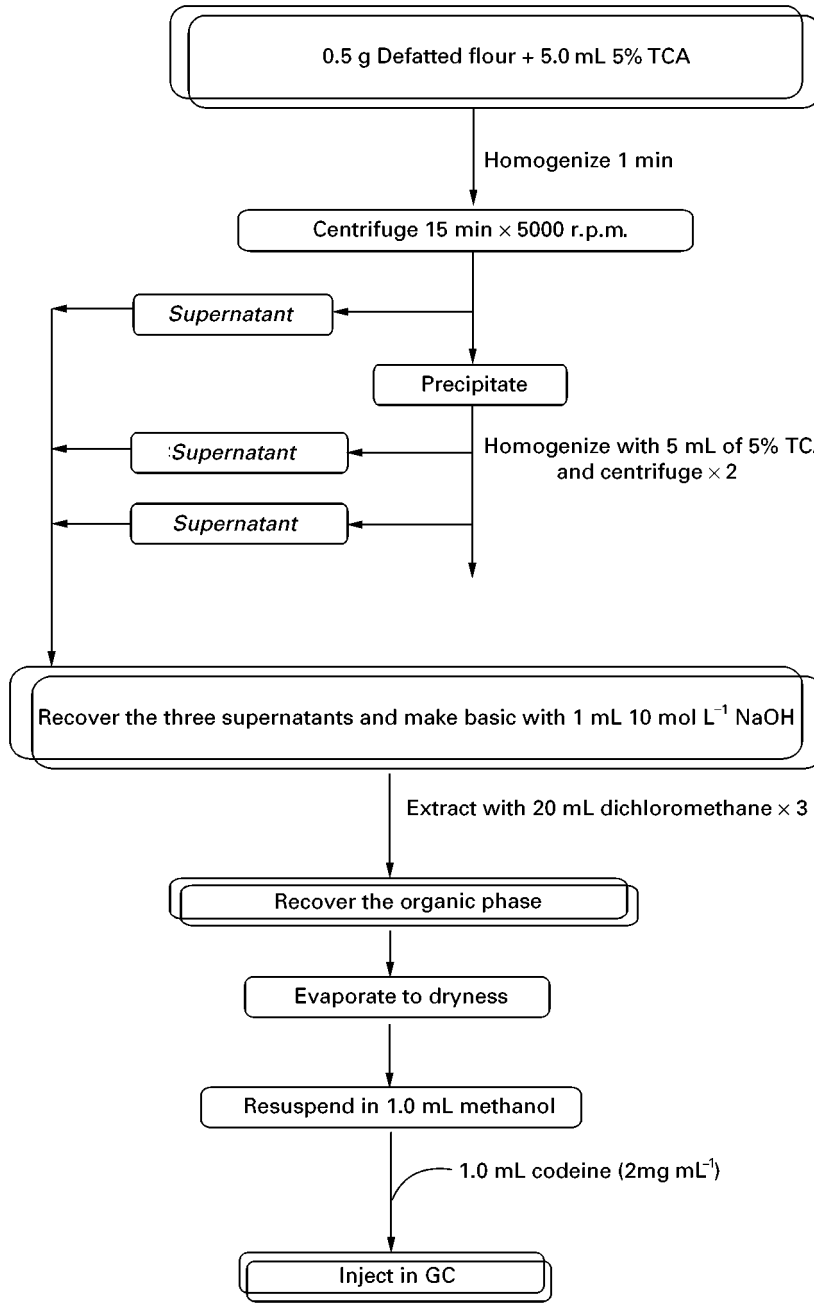
0.5 g Defatted flour + 5.0 mL 5% TCA
Homogenize 1 min
Centrifuge 15 min × 5000 r.p.m.
Supernatant
Precipitate
Supernatant
Homogenize with 5 mL of 5% TCA and centrifuge × 2
Supernatant
Recover the three supernatants and make basic with 1 mL 10 mol L^{-1} NaOH
Extract with 20 mL dichloromethane × 3
Recover the organic phase
Evaporate to dryness
Resuspend in 1.0 mL methanol
1.0 mL codeine (2mg mL^{-1})
Inject in GC

Figure 3 Extraction of quinolizidine alkaloids (Muzquiz *et al.* 1994).

current interest in using analytical-scale SFE stems from the need to replace conventional liquid extraction methods with sample preparation methods that are more efficient, easier to automate, faster and safer to use. Many of the properties of supercritical fluids such as carbon dioxide have facilitated advances in these areas. Thermally labile compounds can be extracted at low temperatures and greatly reduced extraction times. Extracts can also be analysed online by coupling the SFE directly with a gas chromatograph (SFE-GC).

Determination of Alkaloids by Gas–Liquid Chromatography–Mass Spectrometry

Methods for the unequivocal identification and quantification of alkaloids in various, often complex, matrices are of great interest. For this purpose chromatography is widely used. Originally thin-layer chromatography (TLC) was the major method applied for both qualitative and quantitative analysis of alkaloids. Although TLC is still a major tool in alkaloid analysis, in recent years high performance liquid

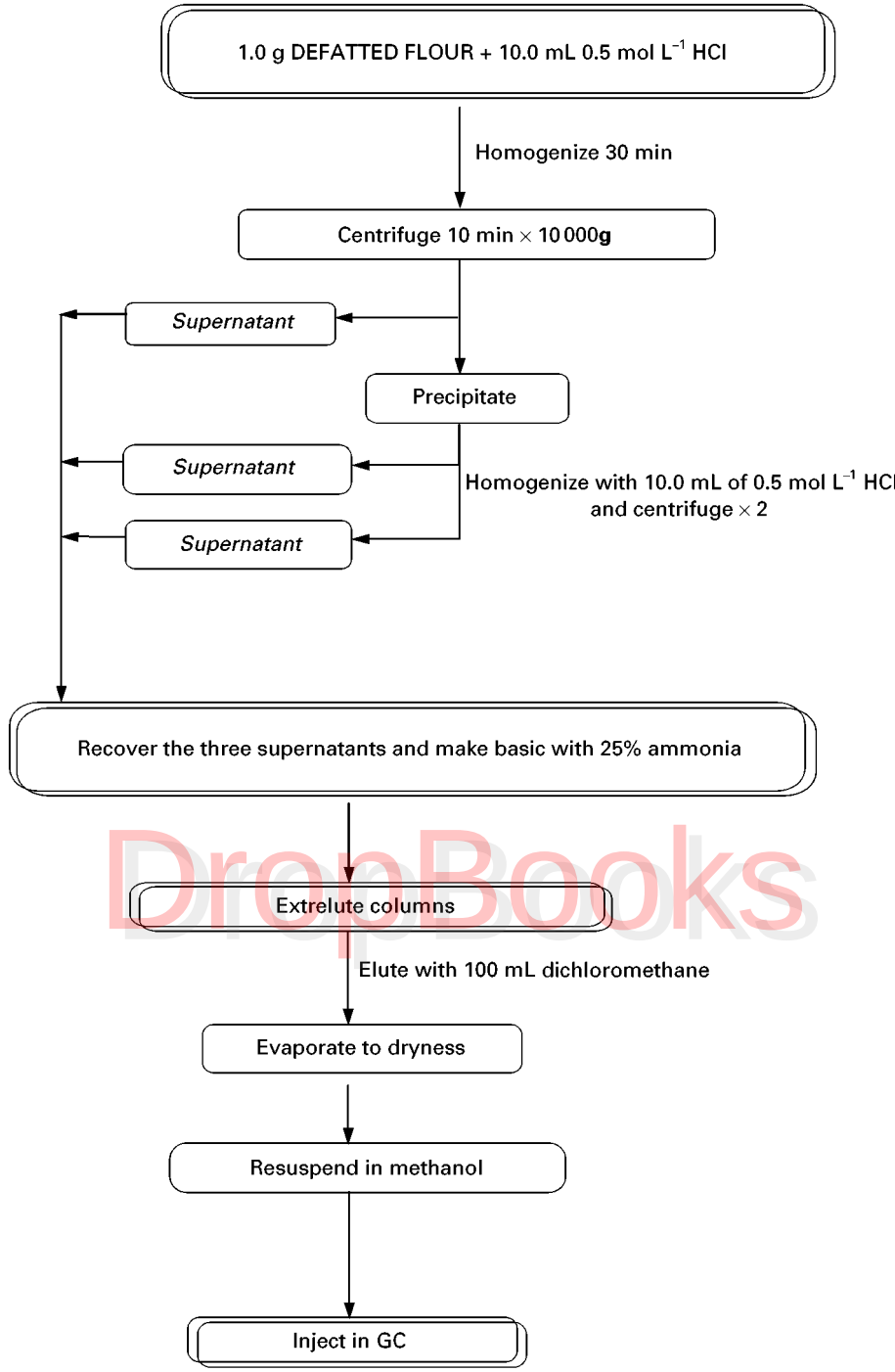
1.0 g DEFATTED FLOUR + 10.0 mL 0.5 mol L^{-1} HCl
Homogenize 30 min
Centrifuge 10 min × 10 000g
Supernatant
Precipitate
Supernatant
Homogenize with 10.0 mL of 0.5 mol L^{-1} HCl and centrifuge × 2
Supernatant
Recover the three supernatants and make basic with 25% ammonia
Extrelute columns
Elute with 100 mL dichloromethane
Evaporate to dryness
Resuspend in methanol
Inject in GC

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Figure 4 Extraction of quinolizidine alkaloids (Wink *et al.* 1995).

chromatography (HPLC) has also developed as an important method for the quantitation of alkaloids. However, more and more applications of capillary (CGC) for complex alkaloids have been reported recently.

Combined gas chromatography–mass spectrometry (GC-MS) has been increasingly used over the last decade for the convenient analysis of alkaloids. This sensitive technique is applicable to the qualitative analysis of individual components of crude alkaloid fractions and is normally able to resolve alkaloid diastereoisomeric pairs. GC-MS is particularly suitable for work of a chemotaxonomic nature, since in such studies it is desirable to identify all the alkaloids

that may have accumulated at a specific time and site in a specific part of a species, rather than only the most abundant compounds present. Also, the use of GC-MS may enable the experimenter to rule out the presence of a particular alkaloid group in the plant material being examined. Impressive separations of alkaloids have been obtained using the high column efficiencies achieved in CGC.

Until 1980 most GLC applications for separating alkaloid mixtures involved packed columns. However, better results can be obtained using the new generation of fused silica capillary columns with bonded phases. The advantage of using small internal diameter columns is not only the higher plate number per unit length, but also the improved lower level of detection due to reduced band broadening. Much more important than efficiency however, is the selectivity that can be introduced into the chromatographic system. The reason for this is that even the best capillary column still has a limited peak capacity (maximum 1000), which is certainly insufficient for unravelling the complex profiles that have to be dealt with in natural product research.

Some GC systems used for the analysis of alkaloids are indicated in **Table 1**. The column selectivity can be adapted to the specific problem by selecting the most suitable stationary phase. Stationary phase selection, however, has no influence on the peak capacity. In addition to universal inlets such a split, splitless, cool on-column and temperature-programmed vaporization, a number of selective inlets are available in CGC.

In the case of QA the capillary columns used (dimensions 15 m × 0.23 mm to 30 m × 0.32 mm) have a high number of theoretical plates (> 70 000), which allow the separation of complex mixtures and even of enantiomers, epimeric at C11 or C6, such as sparteine and α-isosparteine, lupanine and α-isolupanine, 13-hydroxylupanine and 23-epihydroxylupanine, anagyrine and thermopsine, 13α-tigloyloxylupanine and 13β-tigloyloxylupanine, and of *cis* and *trans* isomers, such as 13α-angeloyloxylupanine and 13α-tigloyloxylupanine, as well as the *trans*- and *cis*-cinnamic acid esters.

As a liquid phase several silicone derivatives (0.1 μm or 1 μm films) are employed; good resolutions have been obtained using DB-1 or DB-5 columns, but equivalent products of other manufacturers also work. Split injection techniques are usually appropriate. On-column injection does not provide significant advantages for most applications.

Helium is routinely used as carrier gas, but hydrogen or nitrogen will also work. The injector temperature is usually set at 250°C, that of the detectors at 300°C. Furthermore, even nanogram amounts of alkaloids can be detected by the FID (flame ionization detector) or more sensitively and specifically by a nitrogen-specific detector (NPD).

Hydroxylated QA, such as 13-hydroxylupanine or 3-hydroxylupanine, may be derivatized by trimethylsilyl prior to injection to avoid tailing and to achieve better quantification. Care should be taken not to use the NPD for these derivatives, since the detector would soon be destroyed.

Some authors give relative retention indices for QA. However, Kováts retention indices (RI) give better comparative information and are helpful in identifying individual alkaloids in a GC profile.

Additionally, since this method can be combined with mass spectrometry (GC-MS) it is easy to identify the individual compounds present. Among the spectroscopic methods, mass spectroscopy is definitely the most powerful technique and should therefore take an important place in any laboratory. The problems of interfacing both techniques have been completely

Table 1 Some GC systems used for the analysis of alkaloids

Alkaloid	*Column type, length (m) × i.d. (mm)*	*GC conditions*			*Carrier gas*
		Injector	*Temperature program (°C)*	*Detector*	
Pyrrolizidine	WCOT DB-1 25 m × 0.25 mm	250°C	120–290 (8°C min^{-1})	FID, NPD	He
Quinolizidine	SPB-1 30 m × 0.25 mm	240°C	150–235 (5°C min^{-1})	FID, NPD	He
Tropane	DB-1 15 m × 0.25 mm	250°C	150–270 (6°C min^{-1})	FID, NPD	He
Morphinan	DB-5 15 m × 0.25 mm	280°C	180–270 (30°C min^{-1})–320° (40°C min^{-1})	MSD	He
Aconitum	DB-5 15 m × 0.25 mm	320°C	250–320 (16°C min^{-1})	MSD	He
Amaryllidaceae	DB-1 15 m × 0.25 mm	260°C	200–250 (4°C min^{-1})	FID, NPD	He
Solanum	RT_x-1 15 m × 0.53 mm	270°C	210–260 (1°C min^{-1})	FID	He
Ephedra	HP-5 25 m × 0.20 mm	220°C	90–124 (3°C min^{-1})–280 (20°C min^{-1})	NPD	He

WCOT, Wall coated open tubular column.

overcome by direct coupling (no interface) or the use of an open split interface. Low and high resolution mass spectrometers are the most universal detection devices for CGC. They are capable of electron impact or chemical ionization and can be operated in the full scan mode for identification of unknowns or in the ion-monitoring mode for quantification of target compounds.

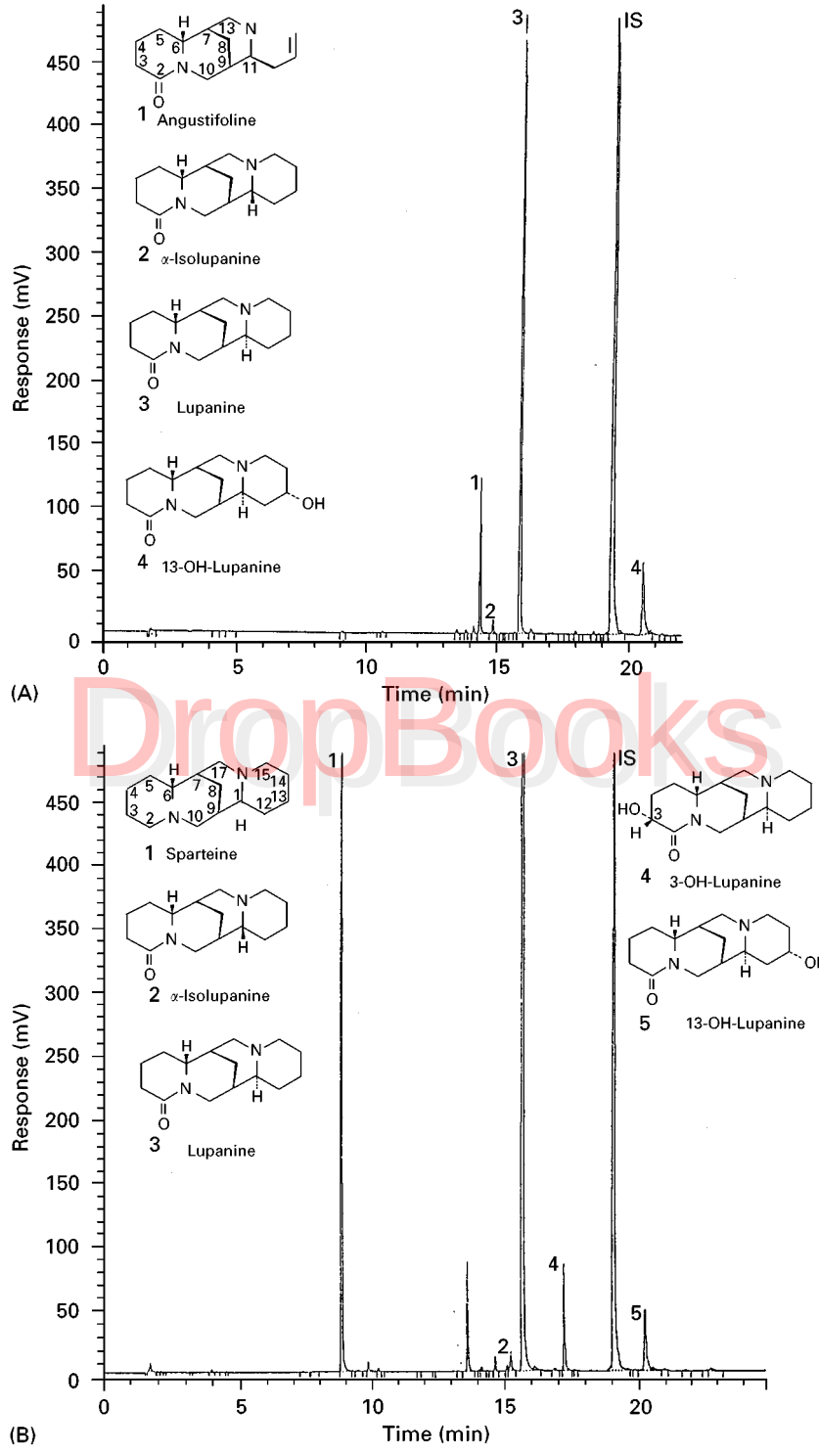

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Figure 5 Separation of an alkaloid extract from *L. angustifolius* (A) and *L. mutabilis* (B) bitter seeds by capillary GC. Injector, 240°C; detector 300°C; oven 150–235°C, 5°C min^{-1}; carrier gas, helium; detection of alkaloids by nitrogen-specific detector (NPD) and mass-selective detector.

Mass spectrometry is widely used today, since QA usually provide distinctive fragmentation patterns in the electron impact mode (EI-MS). Chemical ionization (CI-MS), field desorption (FD-MS) and fast atom bombardment (FAB-MS) are suitable for identifying molecular ions of QA esters and of

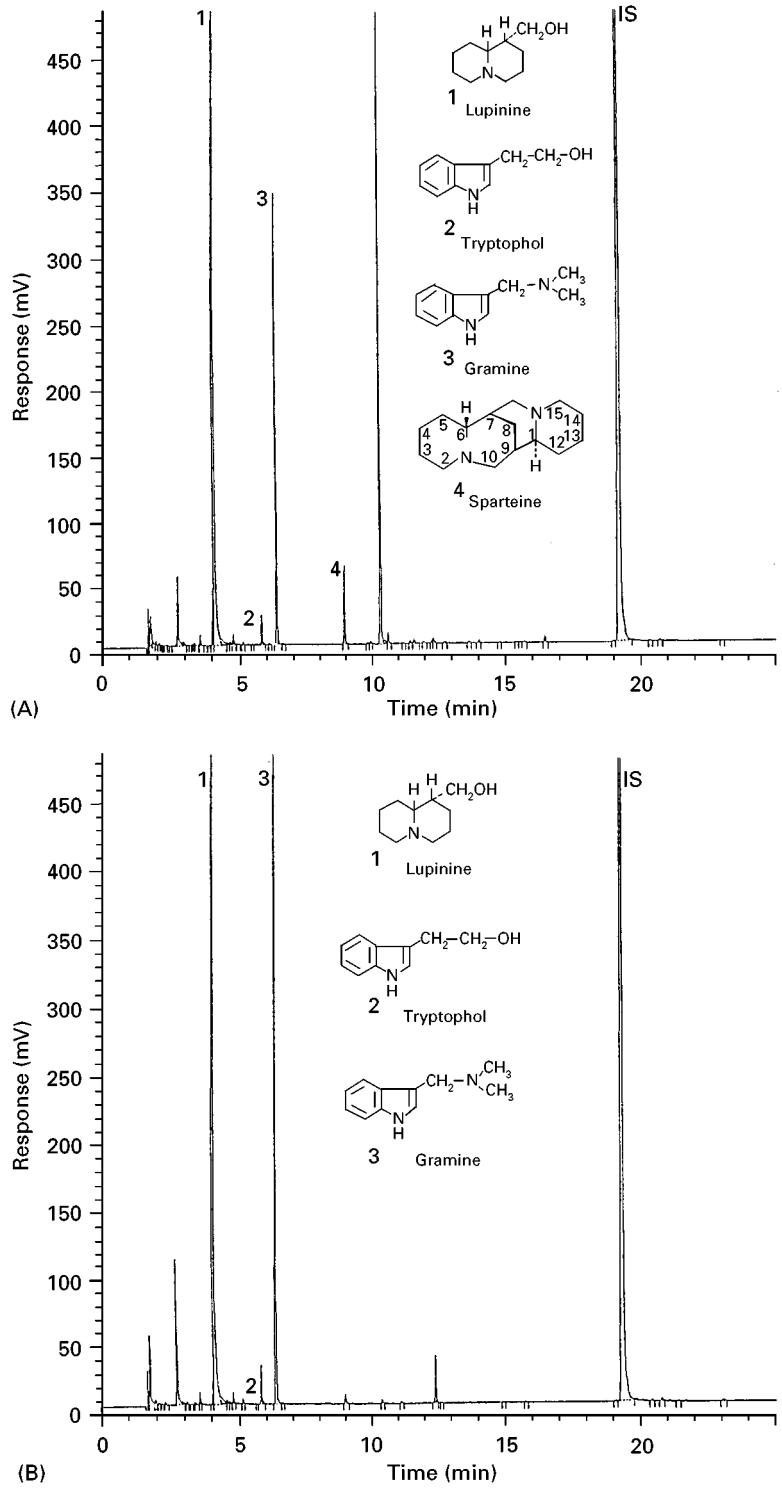

Figure 6 Separation of an alkaloid extract from *L. luteus* (A) and *L. hispanicus* (B) bitter seeds by capillary GC. Injector, 240°C; detector 300°C; oven 150–235°C, 5°C min^{-1}; carrier gas, helium; detection of alkaloids by nitrogen-specific detector (NPD) and mass-selective detector.

tricyclic alkaloids, whose molecular ions are usually obscure or absent in EI-MS spectra. A major advantage of MS is the possibility it gives of combining the high resolution power of capillary GC with the sensitivity of and information provided by EI- or CI-MS. Work using GC-MS was very much facilitated after 1980 by the development of new GC capillary columns, the development of new methods to position the GC column exit near the MS ion source and, most importantly, by improved data processing.

Alkaloid extracts of many legumes contain piperidine alkaloids such as ammodendrine, *N*-methylammodendrine, hystrine or smipine. These alkaloids also derive biogenically from lysine via cadaverine. Simple indole and quinolizidine alkaloids, such as gramine and lupinine may also be encountered. Even combinations of both indole and quinolizidine units are possible, as in the case of *Lupinus hispanicus*.

Separation and identification of QA by GC-MS is shown in **Figures 5–7**.

Conclusion and Future Developments

Gas chromatography is a versatile tool in the analysis of natural products with a wide area of application. It is capable of extracting a wide range of diverse compounds from a variety of sample matrices.

Clear advantages of GC are the high sensitivity of the most common detection method, the FID, and the fact that the detector response of similar compounds will be about the same (i.e. peak areas may be directly compared for quantification). By using a nitrogen-specific detector (NPD) sensitivity for alkaloids can be even further improved while at the same time introducing selectivity.

No systematic studies to determine which column is best suited for alkaloid analysis have been reported, but from the methods described to date it is clear that thinly coated apolar columns are preferred for the analysis of underivatized alkaloids. The length of the columns used varies considerably and it is advisable to test the stability of a compound under GC conditions with a short column. A longer column may be used later if the desired chromatographic resolution has not been achieved.

A wealth of information can be obtained by the analysis of alkaloids by GC coupling to MS. Coupled techniques (GC-MS) have demonstrated their analytical potential. The large amounts of data produced by capillary GC, especially when coupled to a mass spectrometer, can now be handled by a personal

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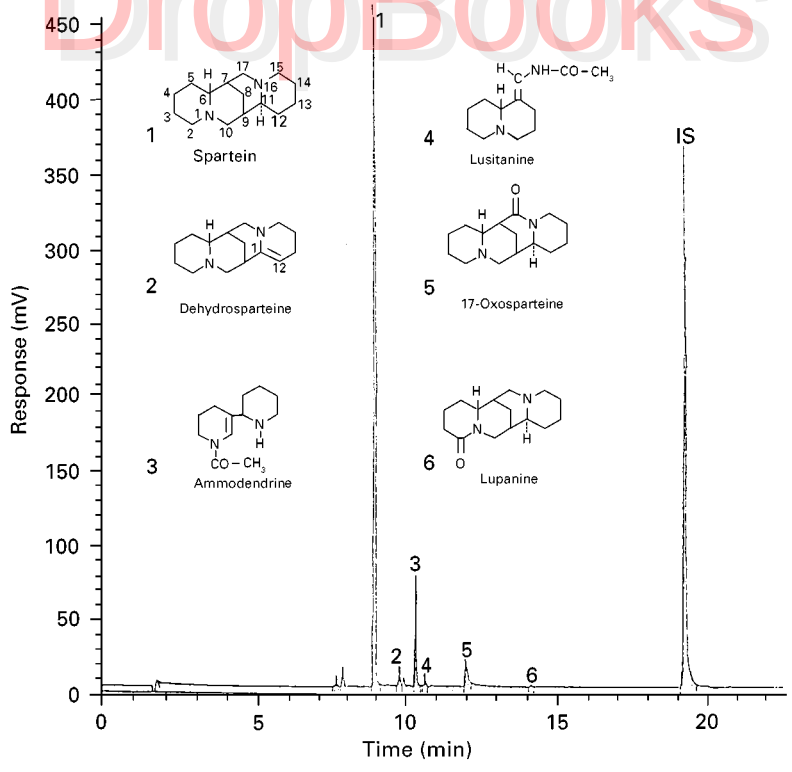

Figure 7 Separation of alkaloid extracts from *Chamaecytisus proliferus* by capillary GC. Injector, 240°C; detector 300°C; oven 150–235°C, 5°C min^{-1}; carrier gas, helium; detection of alkaloids by nitrogen-specific detector (NPD) and mass-selective detector.

computer. The data can be acquired, manipulated and displayed in real time and can be stored for record purposes.

Looking to the future, it is reasonable to expect continued evolutionary development: new selective detectors, more complex analysers for automated sample processing, increasing use of coupled techniques, columns with immobilized phases of a wider range of selectivity, etc. It is hoped that further research and development will encourage the use of GC-MS in the areas of alkaloid analysis that still await investigation.

Acknowledgements

The author gratefully acknowledges M. Martin-Pedrosa, T. Ortega, C. Cuadrado and C. Burbano for their helpful comments.

See also: **II/Chromatography: Gas:** Detectors: General (Flame Ionization Detectors and Thermal Conductivity Detectors); Detectors: Mass Spectrometry; Detectors: Selective. **III/Alkaloids:** Liquid Chromatography; Solid-Phase Extraction; Solid-Phase Microextaction; Supercritical Fluid Extraction; Thin-Layer (Planar) Chromatography. Extraction: Analytical Extractions.

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High Speed Countercurrent Chromatography

See **III / MEDICINAL HERB COMPOUNDS: HIGH SPEED COUNTERCURRENT CHROMATOGRAPHY**

Liquid Chromatography

R. Verpoorte, Leiden/Amsterdam Center for Drug Research, Leiden, The Netherlands

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Definition and Classification of Alkaloids

Alkaloids represent a wide variety of chemical structures (**Figure 1**). More than 16 000 are known and most are derived from higher plants. Alkaloids have also been isolated from microorganisms, marine organisms like algae, dinoflagellates and puffer fish and terrestrial animals like insects, salamanders and toads.

An alkaloid has been defined by Pelletier as a cyclic organic compound containing nitrogen in a negative oxidation state which is of limited distribution among living organisms. From the analytical chemical point of view, the most important trait of alkaloids is their